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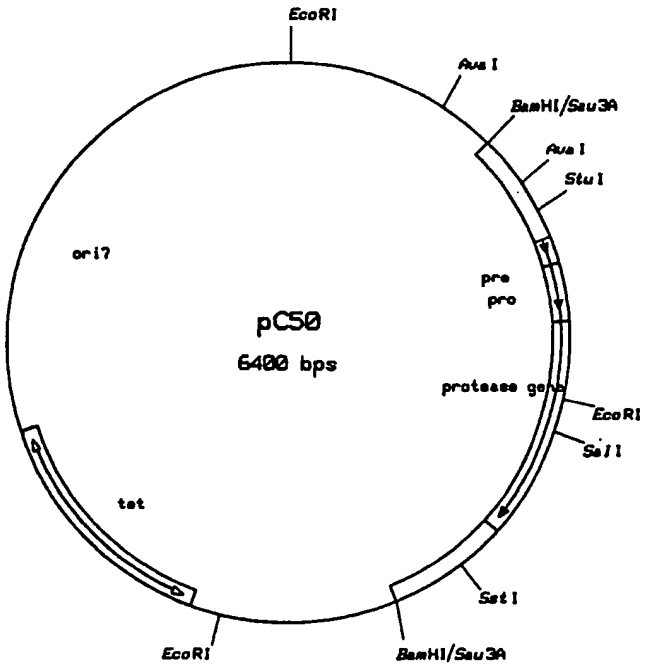
(54) Title: A METHOD OF SUPPRESSING CHROMOSOMAL GENE EXPRESSION

(57) Abstract

A method of inhibiting the expression of a chromosomal protease gene comprising transforming *Bacillus* host with a multicopy plasmid containing a DNA sequence comprising in the direction of transcription a functional gene operably linked to the carboxyl terminal portion of the protease gene.



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A METHOD OF SUPPRESSING CHROMOSOMAL GENE EXPRESSION

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to a method of suppressing the expression of a chromosomal gene in Bacillus by transforming a Bacillus host strain with a multicopy plasmid containing a DNA fragment which suppresses chromosomally encoded protease expression. The invention also relates to multicopy plasmids used for the transformation.

2. Description of the Related Art.

One of the problems associated with the use of Bacillus as extracellular enzyme producers lies in the fact that a number of different proteases are usually produced. Very often at least one of these different proteases is an interfering protease in that it may not have the desired characteristics such as optimum pH, temperature stability, etc, and it may hydrolyze one or more of the other proteases or extracellular proteins produced and excreted by the cell. Interfering protease production is a particularly acute problem in the commercial production of proteases produced by genetically engineered bacteria such as proteases produced for use in laundry detergents. In these cases, chromosomally encoded protease is produced and excreted by bacteria in addition to that produced from the expression of plasmid genes which encode for a

particularly desirable protease. These chromosomally expressed proteases often reduce the yield of the desired protease either through competition for transcription, translation, and/or secretion, or by hydrolyzing the plasmid-encoded protease. It would be advantagous to have a way of repressing or inhibiting chromosomal encoded protease expression in such instances. It would be particularly advantageous to suppress chromosomal gene expression in industrial bacterial strains because no such method exists other than gene inactivation or deletion. Attempts to utilize these methods in classical industrial Bacillus strains has not met with much success. There is a method that selectively enhances the expression of a selected gene disclosed in U.S. patent No. 4,792,523. The patent does not disclose any way of repressing gene expression.

SUMMARY OF THE INVENTION

The present invention provides a method of inhibiting the expression of a chromosomal protease gene comprising transforming a Bacillus host with an multicopy plasmid containing a DNA fragment which suppresses chromosomally encoded protease expression. The present invention also provides multicopy plasmids capable of replicating in Bacillus which comprise a DNA fragment comprising a functional gene linked to the carboxyl terminal portion of the Bacillus licheniformis ATCC 53926 protease gene.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a restriction map of plasmid pC50.

Figure 2 is a restriction map of plasmid pKL1.

Figure 3 is a restriction map of plasmid pKL2

Figure 4 is a restriction map of plasmid pKO7

Figure 5 is a restriction map of plasmid pKL2/SS.

Figure 6 is a schematic representation of the various deletions in the B. licheniformis ATCC 53926 gene.

Figure 7 is a schematic diagram for the construction of the plasmids pC50, pKL1, pKL2, pKO7, and pKL2/SS.

Figure 8. is a restriction map of plasmid pH70B.

Figure 9. is a restriction map of plasmid pH70AMY

Figure 10. is a restriction map of plasmid pC51AMY

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein are to be understood as modified in all instances by the term "about".

The present invention provides a general method of inhibiting the expression of a chromosomally encoded protease gene in Bacilli comprising transforming a Bacillus host with an multicopy plasmid containing a DNA fragment which suppresses chromosomally encoded protease expression. The DNA fragment which suppresses the chromosomally encoded protease expression is the carboxyl terminal portion of the functional protease gene. The carboxyl terminal portion of a protease gene is defined as approximately the distal third of the gene. In the case of B. licheniformis ATCC 53926 the distal third is a DNA sequence between approximately the SalI and approximately the SstI restriction sites.

Multicopy plasmids which are useful in the method of the present invention are those which contain DNA sequences which comprise the carboxyl terminal portion of the chromosomally encoded protease gene. For example, if a host organsism produces a chromosomally encoded interfering protease along with a production protease, the amount of the interfering protease can be susbstantially reduced by transforming the host organism with a plasmid which contains a DNA fragment comprising the carboxyl terminal portion of the chromosomally encoded interfering protease gene. The presence of the approximately distal third of the gene in the plasmid suppresses a large percentage of the chromosomally encoded protease production.

The production protease can be different from the chromosomally encoded protease. For example, the production protease can be a Bacillus lentus alkaline

protease produced by a transformed Bacillus licheniformis mutant strain as is disclosed in a copending U.S. patent application. In this case, a multicopy plasmid which encodes for the Bacillus lentus alkaline protease comprised of a DNA fragment which contains in the direction of transcription a Bacillus lentus alkaline protease functional gene linked to the SalI/SstI restriction fragment of the B. licheniformis ATCC 53926 protease gene.

The production enzyme can be something other than a protease. For example, the production enzyme can be an α-amylase produced by a transformed Bacillus licheniformis ATCC 53926 strain. In this case, a multicopy plasmid comprised of a DNA fragment which contains in the direction of transcription a functional gene which encodes for an α-amylase is linked to the SalI/SstI restriction fragment of the B. licheniformis ATCC 53926 protease gene.

While the method of the present invention can be used in any Bacillus strain it is preferred that the strain be Bacillus licheniformis. It is particularly preferred that the strain be mutant Bacillus licheniformis strains identified by the depository numbers ATCC 10716, ATCC 14580, and ATCC 53926, and DSM 641.

While the method of the present invention can be used in the production of any type of enzyme, it is preferred that the enzyme be a protease.

In a preferred embodiment, plasmid pKO7 (Figure 4) a derivative of plasmid pKL1 (Figure 2) is constructed. Plasmid pKL1 is digested with StuI and SalI, and after subsequent phenolization and precipitation, treated with Klenow polymerase under the appropriate buffer conditions. This DNA is ligated with T4 ligase to form plasmid pKO7. Plasmid pKO7 is deleted for the proximal 2/3 of the structural protease gene including part of the promoter region. B. licheniformis ATCC 53926 transformed by plasmid pKO7 produces only about 10% of the protease produced by the wild type strain.

In order to show that the distal third of the

functional gene suppresses chromosomally encoded protease production, various deletion constructs (Figures 6 & 7) were prepared and their relative protease production was compared. A plasmid containing the structural B. licheniformis ATCC 53926 gene in which the distal third of the gene has been deleted was constructed (Figure 7). First, the alkaline protease gene from B. licheniformis ATCC 53926 was cloned into the vector pBC16 which encodes resistance to tetracycline (J. Bacteriol. 133:897-903, (1978)). The resulting construct was plasmid pC50 (Figure 1) which has been described in U.S. patent application Serial Number 06/892,158, filing date 7/30/86. Plasmid pC50 was then digested with AvaI and after phenolization and ethanol precipitation is religated with T4 ligase to give plasmid pKL1. Plasmid pKL1 was digested with AvaI and StuI and treated with Klenow polymerase in the presence of all four deoxyribonucletides in order to fill in the sticky ends generated by AvaI. The reaction mixture was again treated with phenol and the DNA precipitated with ethanol, and religated with T4 ligase to give plasmid pKL2 (Figure 3). The distal third of plasmid pKL2 was deleted by digesting it with SacI first and then with SalI under the appropriate buffers conditions as recommended by the manufacturer. After phenolization and ethanol precipitation, the digested DNA was redisolved in the appropriate buffer and Klenow polymerase added for conversion of the non-compatible protruding 3' (SacI) and 5' (SalI) ends to blunt ends. After another phenolization and ethanol precipitation step, the DNA was redisolved in a buffer appropriate for the ligation reaction and T4 DNA ligase was added for the connection of the blunt ends of the DNA-fragments to give plasmid pKL2/SS (Figure 5). The data for the relative protease production from the various deletion constructs appears in Table 1.

Table 1

STRAIN	RELATIVE PROTEASE PRODUCTION
ATCC 53926	100%
ATCC 53926/pKL2	< 10%
ATCC 53926/pKO7	< 10%
ATCC 53926/pKL2/SS	ca. 65%
ATCC 53926/pBC16	60-80%

The versatility of the method of the present invention can be shown in another preferred embodiment wherein chromosomal DNA expression is suppressed in a strain transformed to contain multiple copies of an α-amylase gene. Specifically, B. licheniformis ATCC 53926 transformed by a plasmid containing a protease gene which has been inactivated by an α-amylase gene produces only about 20% of the protease produced by the wild type strain. The plasmid (Figure 10) contains a structural amylase gene under the control of a B. licheniformis ATCC 53926 alkaline protease promoter and the SalI/SstI restriction fragment of the B. licheniformis ATCC 53926 protease gene. The construction of the plasmid is described in Examples 6 and 7.

All of the above plasmid DNAs except for pKO7 were first transformed into B. subtilis using protoplast regeneration technique (Chang, S. & Cohen, S.N. (1979) Molec. Gen. Genet. 168:111-115.). After verifying the validity of the constructs, plasmid DNAs were purified using CsCl gradients. The plasmids were then transformed into B. licheniformis ATCC 53926. Plasmid pKO7 is transformed diretly into B. licheniformis ATCC 53926 using the protoplast regenerative technique. Transformants were selected on tetracycline-containing plates and the desired constructs were identified by restriction analysis of mini lysate DNA with suitable restriction enzymes. Transformants were cultured in shake flasks at 39°C and the amount of alkaline protease activity produced was determined according to Dupaix, A., Bechet, J. J. & Roncons, C. (1970)

Biochem. Biophys. Res. Commun. 41: 464 using N-CBZ-valin-p-nitrophenylester as substrate.

The following examples are meant to illustrate but not limit the invention.

Example 1

Construction of Plasmid pKL1

Plasmid pC50 was digested with AvaI and -after phenolization and ethanol precipitation- religated with T4 ligase. The ligation mixture was transformed into competent cells of B.subtilis SB202 (Marmur, J., Seaman, E.S. & Levine, J. (1963) J. Bacteriol. 85:461-467). Selection for transformants was performed on nutrient plates containing 15 micrograms tetracycline/ml. Mini lysate plasmid DNA of transformants was isolated and restricted with EcoR1 in order to identify the desired deletion construct missing the approximately 400 bp AvaI fragment of pC50. For transformation of pKL1 into B. licheniformis ATCC 53926 plasmid DNA was isolated on a large scale and purified using a CsCl-gradient. The purified DNA was then transformed into B. licheniformis ATCC 53926 protoplasts and the desired transformants selected on DM3-agar with 15 micrograms tetracycline and identified as described above for the transformation into B.subtilis.

Example 2

Construction of Plasmid pKL2

pKL1 was digested with AvaI and StuI. Restriction enzymes were removed by phenol treatment and subsequent ethanol precipitation. The digestion mixture was then treated with Klenow polymerase in the presence of all 4 deoxyribonucleotides in order to fill in the sticky ends generated by AvaI. The reaction mixture was again treated with phenol and the DNA precipitated with ethanol. Ligation, transformation into B.subtilis SB202 and identification of transformants containing pKL2 (Figure 3) was performed in the same way as described in example 1 for the construction of pKL1. Transformation of pKL2 into B.

licheniformis ATCC 53926 also followed the procedure described in example 1.

Example 3

Construction of pK07

For the construction of pK07 (Fig.4) pKL1 was digested with StuI and SalI. Restriction enzymes were removed by phenol treatment and subsequent ethanol precipitation. The digestion mixture was treated with Klenow polymerase in the presence of all 4 deoxyribonucleotides in order to fill in the sticky ends generated by SalI. The reaction mixture was again treated with phenol and the DNA precipitated with ethanol. After ligation with T4 ligase, the reaction mixture was transformed into protoplasts of B. licheniformis ATCC 53926. Transformants were selected by growth on plates with DM3-agar with 15 micrograms tetracycline. The desired deletion construct was identified by restriction analysis of mini lysate DNA with EcoR1 as described for pKL1 in example 1.

Example 4

Construction of pKL2/88

The plasmid pKL2, which was deleted for an upstream portion of the alkaline protease gene including part of the putative promoter, was used to construct a second deletion in the distal third of the structural gene between restriction sites SalI and SacI. Plasmid pKL2 was digested with SacI first and then with SalI under the appropriate buffers conditions as recommended by the manufacturer. After phenolization and ethanol precipitation, the digested DNA was redisolved in the appropriate buffer and Klenow polymerase added for conversion of the non-compatible protruding 3' (SacI) and 5' (SalI) ends to blunt ends. After another phenolization and ethanol precipitation step, the DNA was redisolved in a buffer appropriate for the ligation reaction and T4 DNA ligase was added for the connection of the blunt ends of the DNA-fragments. Protoplasts of Bacillus subtilis BC92 were transformed with

the ligation mixture and transformants were selected on DM3 regeneration agar with 15 micrograms/ml tetracycline and identified by restriction analysis of mini lysates. The construct was designated pKL2SS. For transformation of pKL2/SS into B. licheniformis ATCC 53926, plasmid DNA was isolated on a large scale and purified using a CsCl-gradient. The purified DNA was then transformed into B. licheniformis ATCC 53926 protoplasts and the desired transformants selected on DM3-agar with 15 micrograms tetracycline and identified as described above for the transformation into B.subtilis.

Example 5

Expression of subtilisin in Derivatives of B.licheniformis ATCC 53926.

B. licheniformis ATCC 53926 containing the deletion constructs pKL1, pKL2, pK07 or pKL2/SS respectively was cultured in shake flasks in the presence of 7.5 g tetracycline/ml in a complex medium suitable for protease expression (2.4 g/l KH_2PO_4, 1 g/l $MgSO_4$ x $7H_2O$, 0.5 g/l $MnSO_4$ x $2H_2O$, 0.2 g/l $CaCl_2$ x $2H_2O$, 3 g/l soybean flour, 12 g/l casein {Hammarsten}, 120 g/l amylase-treated potatoe starch). At time intervals aliquots were taken for measurement of protease activity. The protease assay was performed using N-CBZ-valin-p-nitrophenylester as substrate and the rate of increase in absorbance at 340nm due to release of p-nitrophenol by the action of protease was determined. Whereas introduction of pC50 or pKL1 into B. licheniformis ATCC 53926 led to overproduction of B. licheniformis ATCC 53926 protease as compared to B. licheniformis ATCC 53926 without plasmid, the productivity of the deletion constructs pKL2 and pK07 was less than 10% relative to the B. licheniformis ATCC 53926 parent. Plasmid pKL2/SS missing the distal sequences between the SalI and SstI sites caused expression of approximately the same level of protease as the plasmid vector without insert DNA (pBC16). (Table 1)

Example 6

Construction of pH70 AMY

The plasmid pJ06 was restricted with BclI, which cuts between the ribosome binding site and the translational start of the α-amylase gene, and at a second site located downstream of the structural α-amylase gene. The plasmid pH70B was restricted with Bcl1 also, but under partial conditions. This plasmid is a derivative of pH70 and contains a BclI site subcloned from pMG56. BclI-cut pJ06 and pH70B were phenolized, ethanol precipitated, and ligated by the action of T4-DNA ligase.

Competent cells of B. subtilis BD393 were transformed with the ligation mixture and selected on Kanamycin containing plates (10 microgram/ml), with an overlay of 1% cornstarch. Transformants which produced clearing zones in the overlay were expected to contain the amylase gene cloned into pH70B. These transformants were verified by analysis of restriction digests of plasmid DNA prepared by a mini-lysate procedure. Those that contained the right construct were identified (amylase gene cloned downstream of the protease promoter, and under its control with the remainder of the protease gene downstream of the transcription putative transcription terminator of the amylase gene). This construct was designated pH70 AMY. Plasmid-DNA was prepared from B. subtilis BD393 pH70 AMY.

Example 7

Construction of pC51 AMY

Both pH70 Amy and pC51 were cut with BamHI and SacI, phenolized, ethanol-precipitated and ligated with T4-DNA-ligase. Competent cells of B. subtilis BD393 were transformed with the ligation mixture and transformants selected on tetracycline containing plates (15 micrograms/ml) with a cornstarch overlay. Clones which produced clearing zones were identified, and verified, by restriction analysis of plasmid DNA from mini lysates. The

correct construct was designated as pC51 AMY. pC51 AMY DNA was purified and used to transform protoplasts of B. licheniformis ATCC 53926.

Example 8

Expression of Subtilisin in B. licheniformis ATCC 53926

B. licheniformis ATCC 53926 containing the deletion construct PC51 AMY was cultured in shake flasks in a complex medium containing 7.5 ug tetracycline/ml. In this complex medium, Plasmid pC51 AMY, which carries the B. licheniformis ATCC 53926 alkaline protease gene inactivated by insertion of the promoterless α-amylase gene, caused a suppression of the protease of B. licheniformis ATCC 53926 to a level of 20% of the level characteristic of the wild type strain.

Experimental Methods

The following methods mentioned in the text were performed essentially as described by T. Maniatis, E.F. Fritsch & J. Sambrook et al. (1982), Molecular Cloning, A Laboratory Manual:

* phenol treatment
* ethanol precipitation
* Klenow polymerase treatment (fill-in reaction)
* ligation with T4 ligase

Mini lysate DNA was prepared according to Birnboim, H.C. & Doly, J. (1979) Nucl. Acids Res. 7:1513.

Large scale plasmid DNA preparation was accomplished essentially as described by Godson, G.N. & Vapnek, D. (1973) Biochem. Biophys. Acta 299:516.

Protoplast transformation was carried out as described by Chang, S. & Cohen, S.N. (1979) Molec. Gen. Genet. 168:111-115.

Preparation of competent cells and their subsequent transformation followed the protocol of Cahn, F.H. & Fox, M.S. (1968) J. Bacteriol. 95:867-875.

Plasmids

Plasmid pJ06 is described in German Patent Application 3824827.

Plasmid pC50 is described in U.S. patent application Serial Number 06/892,158 filed on July 30, 1986.

Plasmid pH70B. Plasmid pH70B is a derivative of plasmid pH70 (DSM 5479). Plasmid pMG56 was cut with EcoRI and then with BamHI. pH70 was cut with BamHI and with EcoI under partial cleavage conditions. Both digested plasmids were phenolized, mixed together, and precipitated with ethanol. After ligation with T4 DNA ligase, competant cells of Bacillus subtilis SB202 were transformed and selected on Kanamycin with 10 microgram/ml. Transformants were screened by BclI digests of mini lysate DNA. Clones exhibiting BclI site between the ribosome binding site and the translational start site were designated pH70B.

Deposit of Microorganisms

A living culture of B. licheniformis ATCC 53926 containing plasmid pKO7 has been accepted for deposit in Deutsche Sammlung Von Mikroorgnismen (DSM), Grisebachstr. 8, 3400 Gottingen, Federal Republic of Germany, under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the purposes of patent procedure and has been assigned the identification number DSM 5472.

What is claimed is:

1. A method of inhibiting the expression of chromosomal protease gene comprising transforming a Bacillus host with an multicopy plasmid containing a DNA fragment which suppresses chromosomally encoded protease expression.

2. The method of claim 1 wherein said DNA fragment is a sequence between approximately the SalI and SstI restriction sites of the B. licheniformis ATCC 53926 alkaline protease gene.

3. The method of claim 1 wherein said Bacillus host is Bacillus licheniformis.

4. The method of claim 3 wherein said Bacillus host is Bacillus licheniformis ATCC 10716.

5. The method of claim 3 wherein said Bacillus host is Bacillus licheniformis ATCC 14580.

6. The method of claim 3 wherein said Bacillus host is Bacillus licheniformis ATCC 53926.

7. The method of claim 3 wherein said Bacillus host is Bacillus licheniformis DSM 641.

8. An multicopy plasmid capable of replicating in Bacillus containing a DNA fragment comprising a functional gene linked to the SalI/SstI restriction fragment of the B. licheniformis ATCC 53926 protease gene..

9. The multicopy plasmid of claim 8 wherein said functional gene is the B. licheniformis ATCC 53926 protease gene.

10. The multicopy plasmid of claim 8 wherein said functional gene is the amylase gene from B. licheniformis ATCC 53926.

11. A multicopy plasmid having the cleavage map substantially as shown in Figure 4.

12. A transformed Bacillus licheniformis host cell comprising a hybrid plasmid of claim 11.

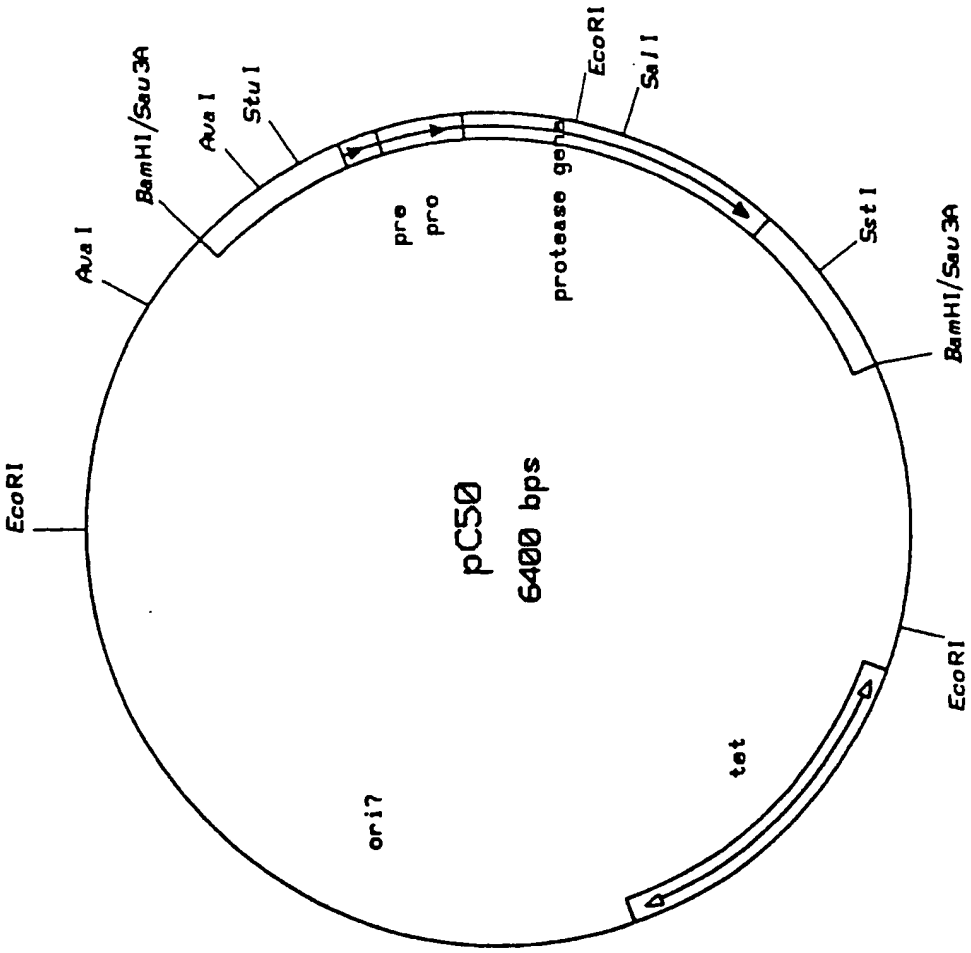
13. A transformed host cell of claim 8 wherein said host cell is Bacillus licheniformis ATCC 10716.

14. A transformed host cell of claim 8 wherein said host cell is Bacillus licheniformis ATCC 14580.

15. A transformed host cell of claim 8 wherein said host cell is Bacillus licheniformis ATCC 53926.

16. A transformed host cell of claim 8 wherein said host cell is Bacillus licheniformis DSM 641.

FIGURE 1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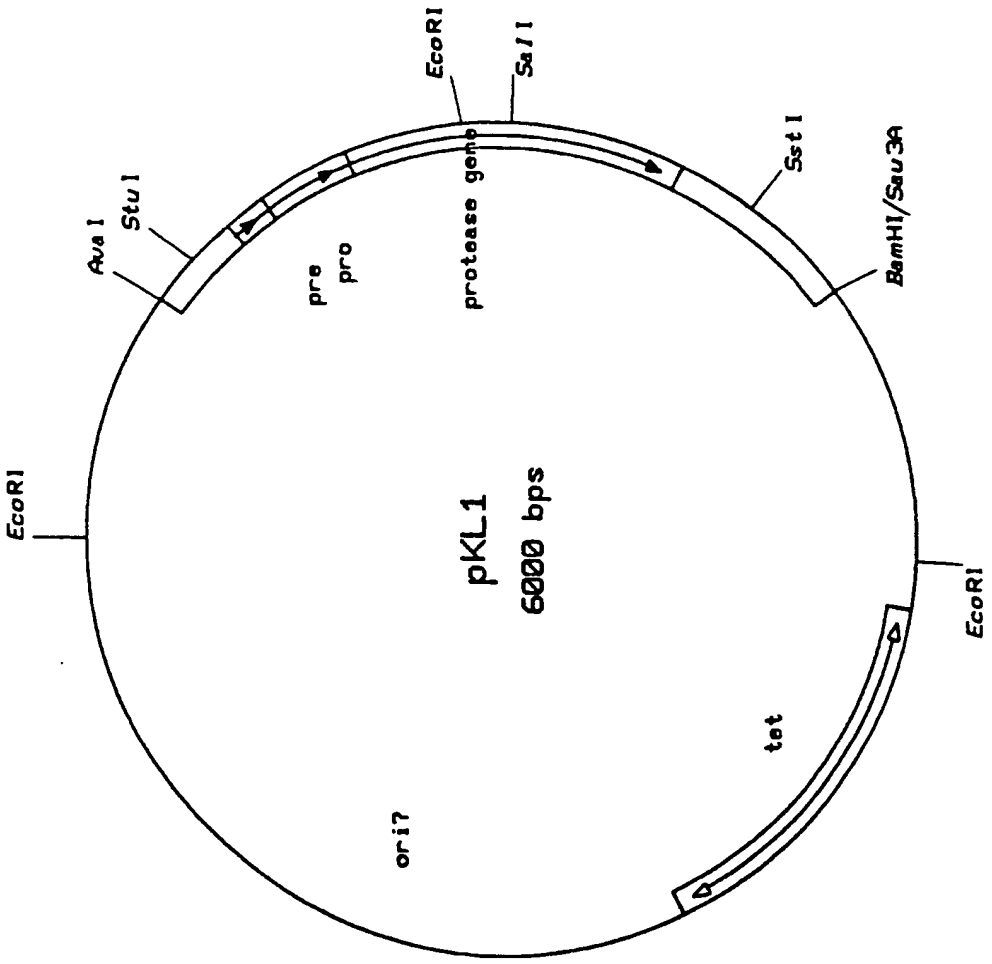
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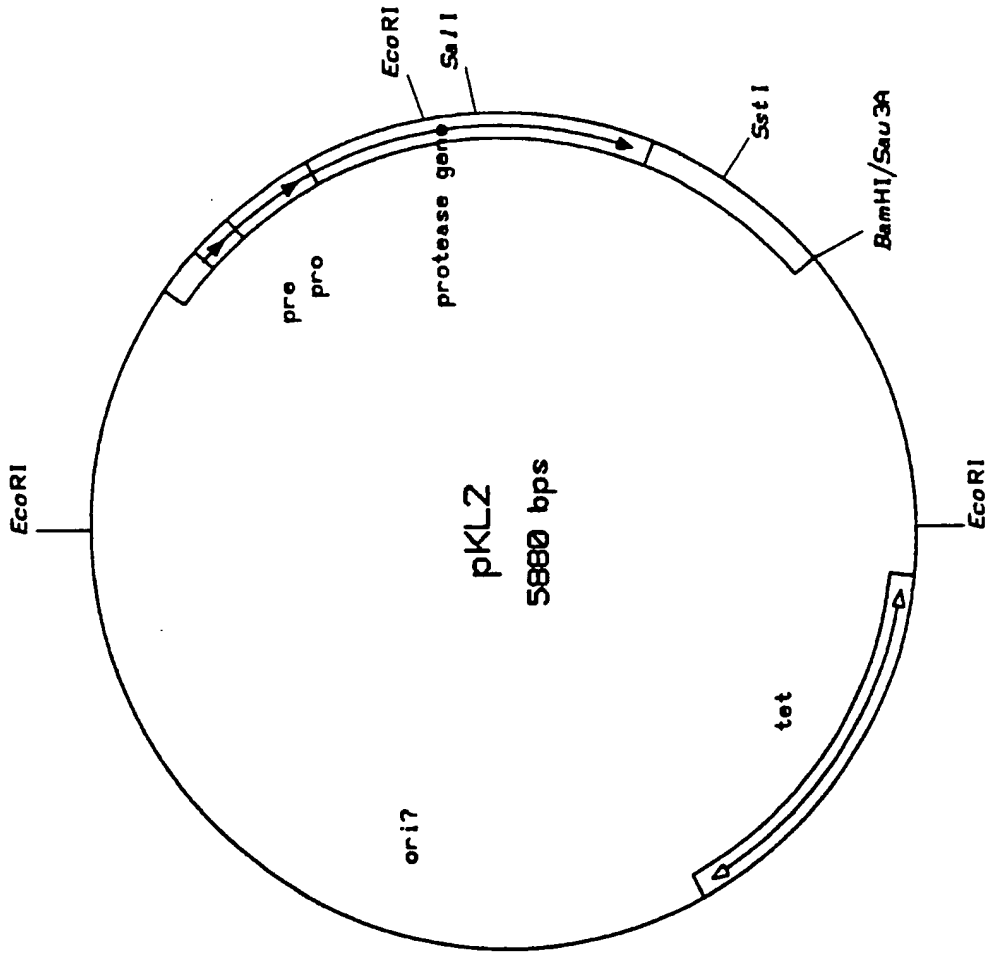
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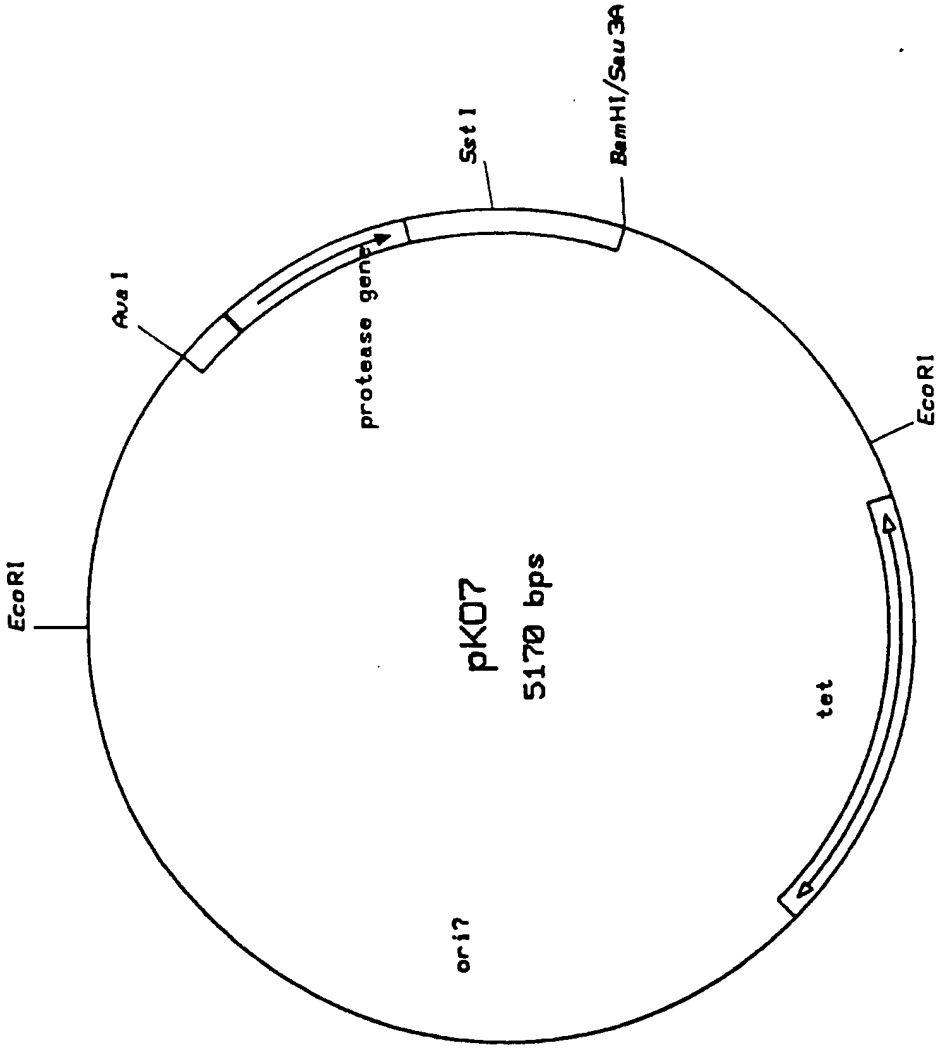
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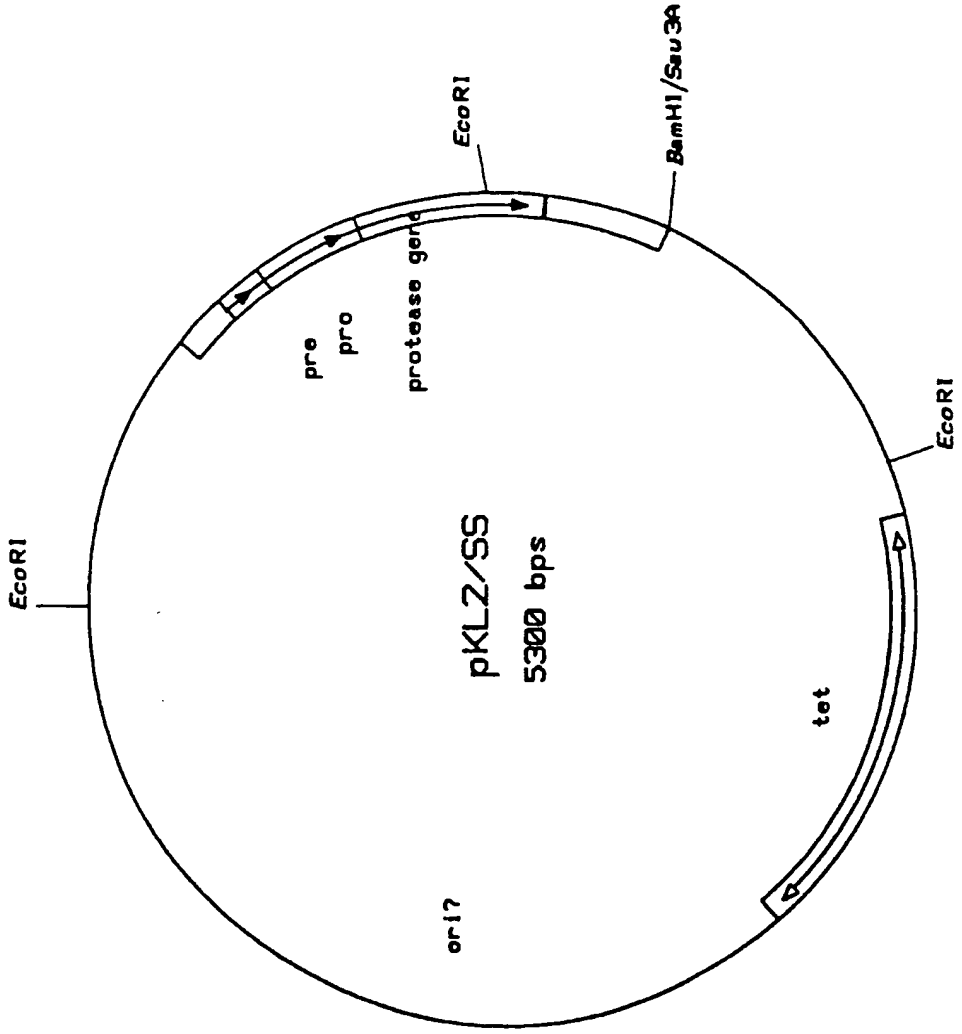
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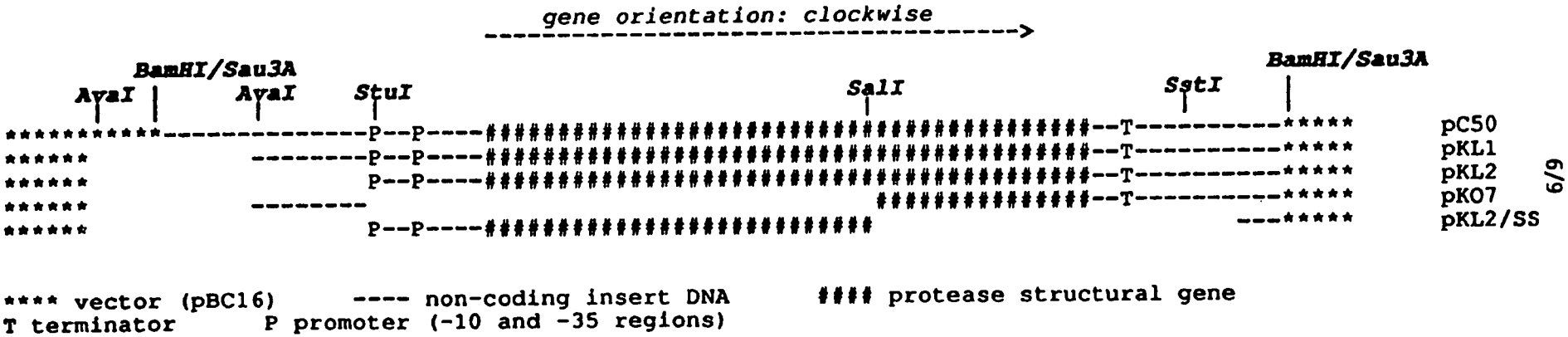
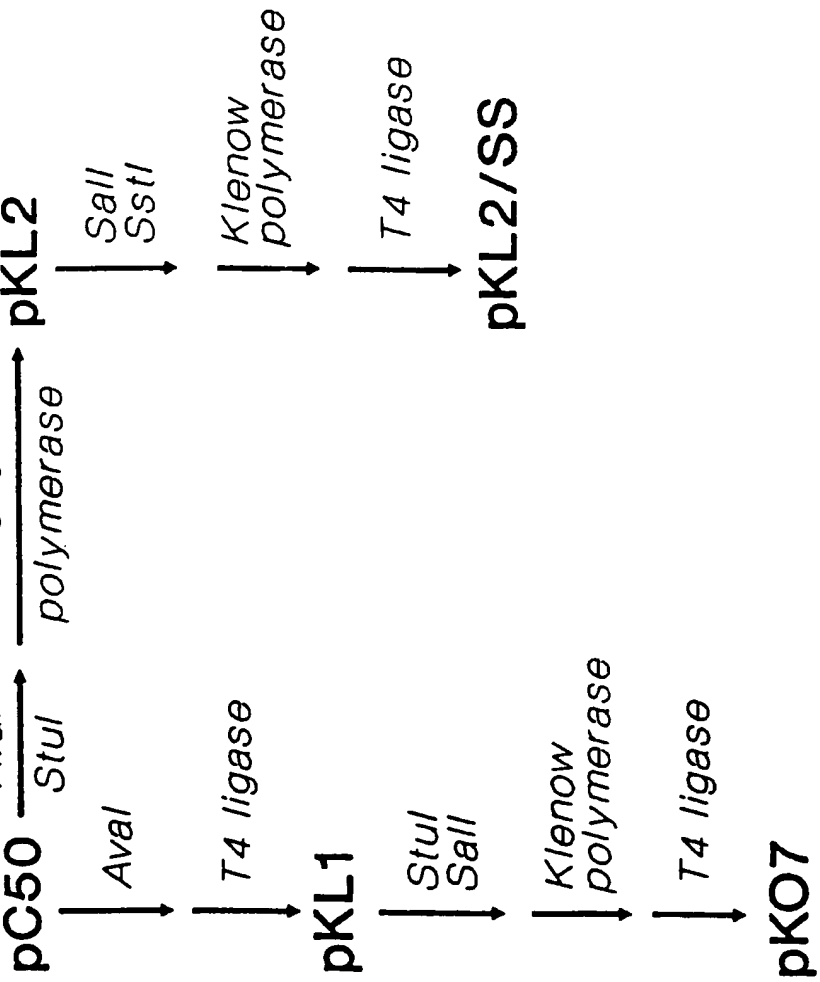


FIGURE 7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StuI
SalI
Klenow polymerase
T4 ligase
pKO7

FIGURE 8

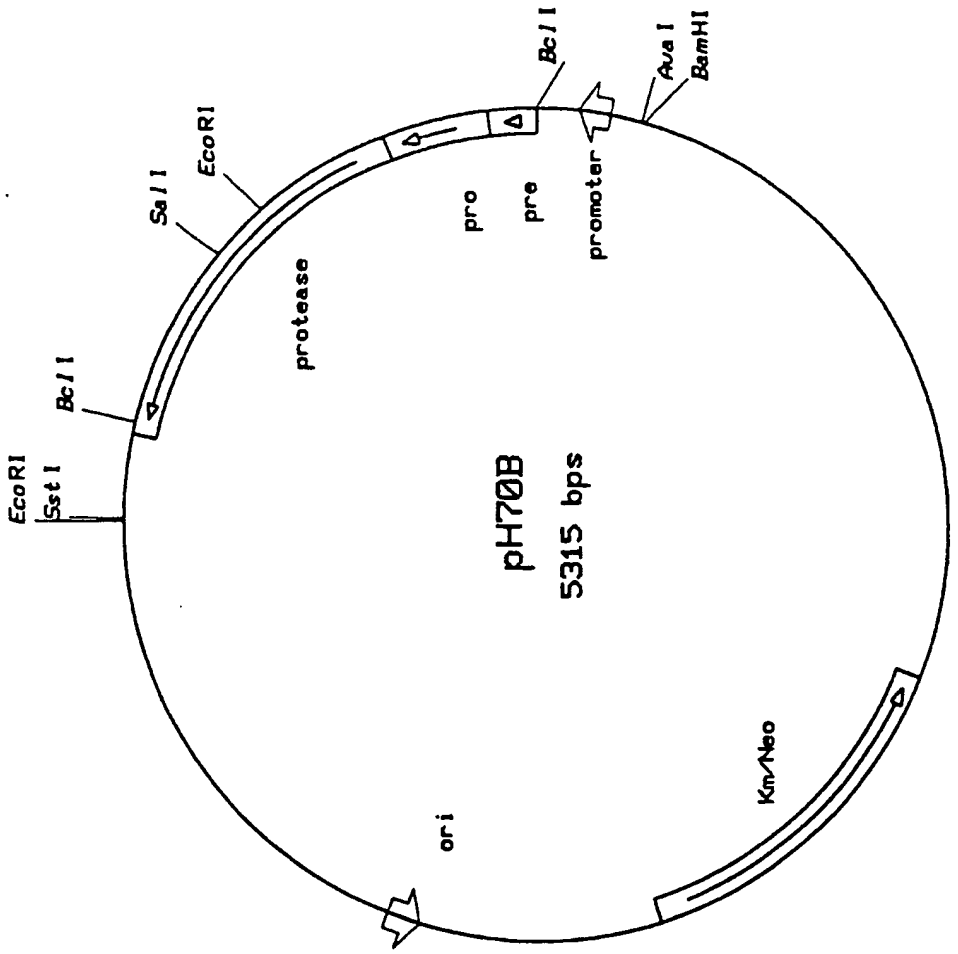
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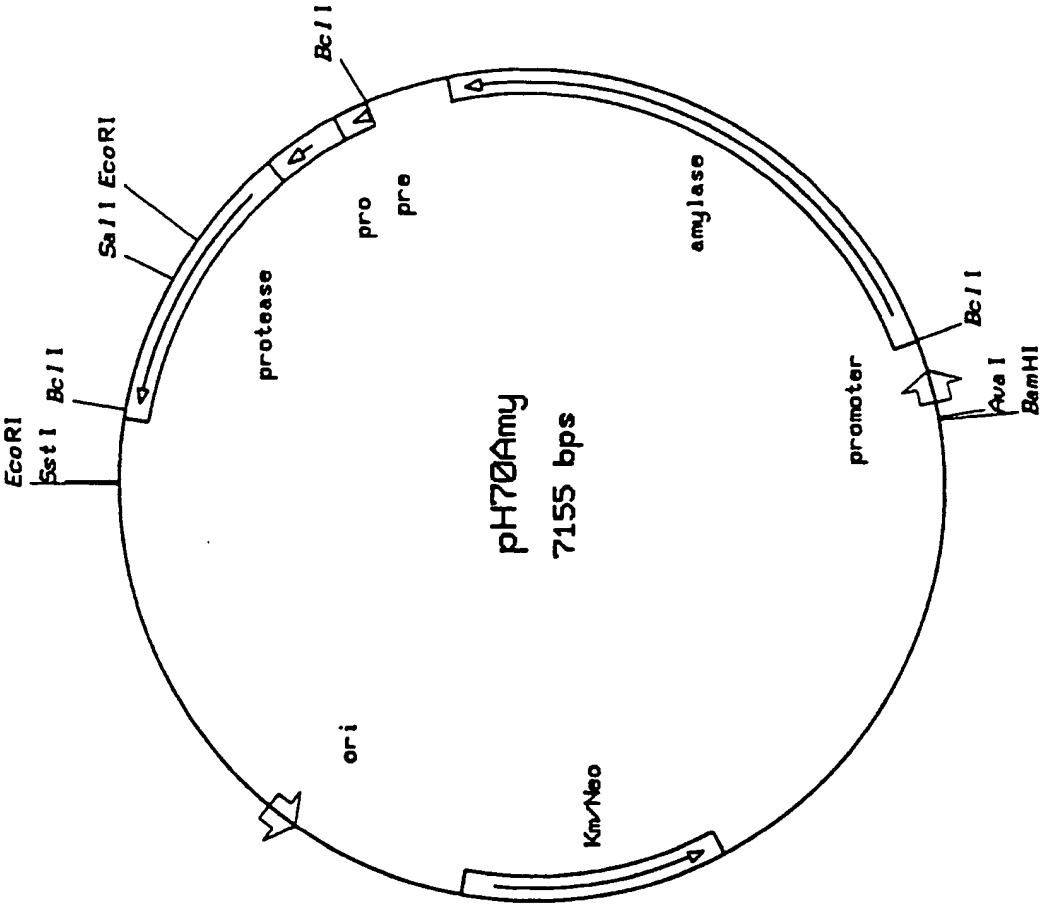
FIGURE 9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Km/Neo

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/04713

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/75, C 12 N 15/57, C 12 N 15/56, C 12 N 1/21, (C 12 N 1/21; C 12 R 1:10)

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Patent Abstracts of Japan, volume 13, no. 367, (C-626)(3715), 15 August 1989 & JP, A, 1124386 (MITSUBISHI KASEI CORP.) 17 May 1989 see abstract	1
X	FR, A, 2604726 (AGENCY OF INDUSTRIAL SCIENCE AND TECHNOLOGY) 8 April 1988 see claims; page 6, line 13 - page 8, line 27	1
A	WO, A, 86/01825 (GENEX CORP.) 27 March 1986 see claims	1
A	EP, A, 0214435 (HENKEL KG) 18 March 1987 see figure 1	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23rd November 1990	20. 12. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	R.J. Eernisse

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9004713 SA 39641

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 17/12/90 The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR-A- 2604726	08-04-88	JP-A- 63087975	19-04-88
		GB-A,B 2198439	15-06-88
WO-A- 8601825	27-03-86	US-A- 4828994	09-05-89
		EP-A- 0195078	24-09-86
		JP-T- 62500215	29-01-87
EP-A- 0214435	18-03-87	DE-A- 3527913	12-02-87
		AU-A- 6078986	05-02-87
		JP-A- 62032888	12-02-87